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ORIGINAL INVESTIGATION

Nicotine-induced enhancement of a sensory reinforcer in adult rats: antagonist pretreatment effects

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Abstract

Rationale and objectives The reinforcement-enhancing effect (REE) of nicotine refers to the drug's ability to enhance the strength of other primary and conditioned reinforcers. The main aim was to investigate neuropharmacological mechanisms underlying nicotine's strengthening of a primary visual reinforcer (i.e., a light cue), using a subcutaneous (SC) dose previously shown to provide plasma nicotine levels associated with habitual smoking.

Methods Adult male rats pressed an "active" lever to illuminate a brief cue light during daily 60-min sessions. Rats that showed a clear REE were tested with systemically administered pretreatment drugs followed by nicotine (0.1 mg/kg SC) or saline challenge, in within-subject counterbalanced designs. Pretreatments were mecamylamine (nicotinic, 0.1-1 mg/kg SC), SCH 39166 (D1-like dopaminergic, 0.003-0.2 mg/kg SC), naloxone (opioid, 1 and 5 mg/kg SC), prazosin (alpha1-adrenergic antagonist, 1 and 2 mg/kg IP), rimonabant (CB1 cannabinoid inverse agonist, 3 mg/kg IP), sulpiride (D2-like dopaminergic antagonist, 40 mg/kg SC), or propranolol (beta-adrenergic antagonist, 10 mg/kg IP).

Results The nicotine REE was abolished by three antagonists at doses that did not impact motor output, i.e., mecamylamine (1 mg/kg), SCH 39166 (0.01 and 0.03 mg/kg), and naloxone (5 mg/kg). Prazosin and rimonabant both attenuated the nicotine REE, but rimonabant also suppressed responding more generally. The nicotine REE was not significantly altered by sulpiride or propranolol.

Conclusions In adult male rats, the reinforcement-enhancing effect of low-dose nicotine depends on nicotinic receptor stimulation and on neurotransmission via D1/D5 dopaminergic, opioid, alpha1-adrenergic, and CB1 cannabinoid receptors.

Keywords Nicotine · Reinforcement enhancement · Smoking · Dopaminergic · Adrenergic · Opioid · Cannabinoid

Introduction

While nicotine generally serves as a weak primary positive reinforcer in drug self-administration studies (Caggiula et al. 2002; Fulton and Barrett 2008; Jensen et al. 2016; Rose et al. 2010), it is the drug's ability to make other reinforcers more powerful that is potentially more relevant to tobacco addiction (Perkins et al. 2017; Rupprecht et al. 2015). This reinforcement enhancing effect (REE) of nicotine has been identified across a range of primary and conditioned reinforcers, including both sensory and non-sensory stimuli, in both rodents (Rupprecht et al. 2015) and human smokers (Perkins et al. 2017).

A nicotine REE has been extensively studied in rats, in the context of nicotine intravenous self-administration (IVSA) (Rupprecht et al. 2015). Here, each drug infusion is paired with the brief illumination of a cue light which is mildly reinforcing in its own right and can also acquire conditioned reinforcing properties. Nicotine-taking behavior is thus supported not only by direct reinforcing effects of the drug itself but also by primary or conditioned reinforcing effects of the light cue, which are in turn amplified through a nicotine REE (Rupprecht et al. 2015). Since nicotine is at best poorly reinforcing in the absence of audiovisual cues (Caggiula et al. 2002; Donny et al. 2003; Palmatier et al. 2006; Sorge et al.

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2009), its role in this behavioral assay seems to be primarily one of the reinforcement enhancement.

Nicotine REEs have been more directly studied, both in rats and human subjects, by testing the influence of *noncontingent* nicotine administration on responding for non-nicotine primary or conditioned reinforcers (Perkins et al. 2017; Rupprecht et al. 2015). In rats, the most commonly employed primary reinforcers are rewarding electrical brain stimulation and weakly reinforcing visual stimuli, whereas conditioned reinforcers typically comprise one or more sensory stimuli that have been previously paired with water or sucrose (Paterson 2009; Rupprecht et al. 2015).

In order to demonstrate a nicotine REE, it is necessary to control for general effects of the drug on motor output. In animal experiments, this has been achieved in two main ways: by using rate-free measures in brain stimulation reward studies (Clarke and Kumar 1984; Paterson 2009) or, where natural reinforcers are used, by comparing response rates on active vs. inactive manipulanda (Constantin and Clarke 2018; Palmatier et al. 2006). Overall, most but not all published studies have revealed a clear nicotine REE independent of locomotor effects (see Constantin and Clarke 2018).

The neuropharmacological basis of nicotine REEs has been partially elucidated. For example, enhancement of responding for brain stimulation reward and conditioned sensory reinforcers appears to depend on the participation of $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors (nAChRs) (Guy et al. 2014; Spiller et al. 2009; Tobey et al. 2012) and several other transmitter systems (see Discussion). For *primary sensory* reinforcers, the focus of the present study, nicotine-induced enhancement is reported to be mimicked by the partial nAChR agonist varenicline (Levin et al. 2012), abolished by the nAChR antagonist mecamylamine (Kirshenbaum et al. 2014; Liu et al. 2007; Palmatier et al. 2009), reversed by the opioid antagonist naloxone (Kirshenbaum et al. 2016), and unaffected by pretreatment with adrenergic or glutamatergic receptor antagonists (Palmatier et al. 2009; Palmatier et al. 2007).

While the term "nicotine reinforcement enhancing effect" suggests a unitary phenomenon, it is also possible that multiple underlying mechanisms exist, depending in part on the level of nicotine exposure. Critically, most nicotine REE studies in adult rats use doses (typically 0.3–0.4 mg/kg SC) that would result in sustained plasma nicotine levels beyond the normal smoking range (discussed in Constantin and Clarke 2018). This is a significant limitation insofar as there appears to be no convincing evidence that rat nAChRs are less sensitive than their human counterparts. Given that nAChR subtypes differ in their sensitivity to nicotine (Rollema and Hurst 2018), high doses of nicotine could potentially engage nAChRs and downstream signaling mechanisms that are of little, if any, relevance to smoking.

To address this concern, we recently identified a REE occurring at lower nicotine doses (i.e., 0.05–0.1 mg/kg SC) which yielded peak serum levels (12–25 ng/ml) that closely match steady-state levels reported in typical smokers (Constantin and Clarke 2018). The present study represents a first pharmacological characterization of this low-dose nicotine REE. As in our recent published work (Constantin and Clarke 2018; Wright et al. 2018), rats were permitted to self-administer a brief visual stimulus (cue light) in a series of simultaneous choices between "active" and "inactive" retractable levers. Most rats pressed preferentially on the active lever, confirming that the visual stimulus was reinforcing, and in addition nicotine selectively increased active lever pressing, demonstrating a REE. With a nicotine REE established, we investigated whether any pretreatment drug, when given alone, blunted the reinforcing effect of the visual stimulus or disrupted responding more generally. Most of our pretreatment drugs were selected because they had been reported to inhibit nicotine IVSA or a higher-dose nicotine REE or both (see Table 1 and Discussion). Pretreatment drugs were first each tested at a single, moderately high dose (Experiment 1), after which a subset was selected for testing at additional doses (Experiments 2–4).

Methods

Animals

Male young adult Long-Evans rats ($N = 174$) were obtained from Charles River, from two locations: St. Constant, QC, Canada, for Experiments 1–3 and Kingston NY, USA, for Experiment 4. Subjects weighed 200–250 g (Experiments 1–3) or 200–225 g (Experiment 4) upon arrival. They were housed 2–3 per cage in a temperature- and humidity-controlled animal colony maintained on a reverse 12:12 light/dark cycle, with lights off at 0700 h. All behavioral testing took place during the dark phase of the cycle, between 0800 h and 1700 h. Food and water were available ad libitum in the home cage. All experimental protocols were approved by the McGill Medical Faculty Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines, in order to minimize pain and discomfort.

Drugs

Drugs and suppliers were (−)-nicotine hydrogen tartrate salt, S(−)sulpiride, and (±)propranolol HCl (Sigma-Aldrich, Oakville ON, Canada); SCH 39166 HBr (Tocris, Oakville ON, Canada); mecamylamine HCl, prazosin HCl, and naloxone HCl (Toronto Research Chemicals, Toronto ON, Canada); and rimonabant-free base (gift from the NIMH Chemical Synthesis and Drug Supply Program, USA).

Table 1 Summary of pretreatment drug conditions

Drug	Dose mg/kg (base)	Route	Pretreat-challenge interval* (min)	Vehicle
Experiment 1				
Mecamylamine	1	SC	20	Saline
SCH 39166	0.2	SC	20	Saline
Naloxone	1	SC	10	Saline
(±)Propranolol	10	IP	20	Saline
S(−)Sulpiride	40	SC	30	Acetic acid (dilute)
Rimonabant	3	IP	20	DMSO/Tween-80
Prazosin	1	IP	20	Water
Experiment 2				
Mecamylamine	0.1, 0.3, 1	SC	20	Saline
Experiment 3.1				
SCH 39166	0.01, 0.03, 0.1	SC	20	Saline
Experiment 3.2				
SCH 39166	0.003, 0.01, 0.03	SC	20	Saline
Experiment 4				
Prazosin	1, 2	IP	20	Saline
Naloxone	1, 5	SC	10	Saline

*Note: Nicotine (0.1 mg/kg SC) or saline was injected after the specified interval, immediately pre-session

Drugs were dissolved in sterile 0.9% saline, except as follows. Prazosin HCl was dissolved in water, with sonication. Rimonabant was dissolved in a vehicle of dimethylsulfoxide (DMSO), Tween-80, and 0.9% saline, in a ratio of 1:2:7. Sulpiride was suspended in saline, and then dissolved by addition of glacial acetic acid (0.8% *v*/v), with the final pH increased to 6.0 by dropwise addition of 5 M NaOH. This DMSO/Tween vehicle was also pH-adjusted to 6.0 before being given alone. Nicotine solutions were adjusted to pH 7.1–7.3 with dilute NaOH. Drugs were administered in a volume of 1 ml/kg except for prazosin HCl (2 ml/kg). Doses of all drugs are expressed as the base. Drug solutions were aliquotted and stored at −20 °C until the day of use. Doses, routes, and times of administration are stated in Table 1. The choice of doses and timing of pretreatment injections was based on the published literature, as documented in **Supplementary Table S1**.

Behavioral apparatus and testing procedure

The apparatus and procedure were unchanged from our previous reports (Constantin and Clarke 2018; Wright et al. 2018). Subjects were tested in operant conditioning chambers (ENV-008CT, Med Associates, Lafayette, IN) housed within melamine cubicles. Each box was equipped with two retractable levers (ENV-112CM) located 10 cm apart and 8 cm above the stainless steel bar floor. A white cue light (2.5 cm diameter, 28 V, 100 mA, ENV-221 M) was situated 3 cm above each of the two levers. A white house light (28 V, 100 mA, ENV-215 M) was located on the opposite wall but was not used. All visual stimuli were controlled by Med Associates software. For each rat, one lever was designated "active" and the other "inactive." The left-right positions of the active and inactive levers were counterbalanced within each group of subjects. An FR1 schedule of reinforcement was used, with a single response on (only) the active lever producing a visual stimulus. This stimulus comprised a 3-s cue light illuminated above the active lever. A response on either lever resulted in the immediate retraction of both levers (i.e., coincident with light cue onset) for a time-out period of 60 s, after which the levers were again extended into the chamber. Hence, rats could obtain almost 60 visual stimuli per 60-min session at maximum.

Nicotine or saline were injected SC immediately before the test session. Pretreatment injections were given by either SC or intraperitoneal (IP) injection 10, 20, or 30 min pre-session, depending on the drug (see Table 1).

Experimental design and timeline

Each experiment comprised the following sequential phases: (1) handling and habituation, (2) acquisition, (3) saline/nicotine testing, and (4) pretreatment/nicotine testing. During handling and habituation (3–5 days), rats were handled for a few minutes each day, tail-marked, and weighed in the colony room. For the rest of the experiment, rats received daily 60-min sessions in the operant chambers. Depending on experiment, the initial task acquisition phase (no injections) lasted

5–8 days, and the subsequent saline/nicotine testing phase lasted 8–12 days. During the latter phase, each rat was tested after injection of nicotine (0.1 mg/kg SC) or saline; these conditions occurred on alternate days, in a counterbalanced manner. On saline test days during this phase, rats received a home cage injection of nicotine 1–4 h post-session. At the end of this phase, rats were selected for further testing, based on three criteria: subjects had to (1) significantly prefer the active lever over the inactive lever, (2) make on average at least 5 active lever presses per session, and (3) press more on the active lever under nicotine than under saline. During the final antagonist/nicotine testing phase, the selected rats were each tested under all possible combinations of antagonist pretreatment and saline/nicotine treatment, within a full or partial Williams square design, in order to counterbalance for carry-over effects as far as possible. Experimenters were blind to drug conditions during testing and analysis.

Experiment 1: Single-dose tests with multiple antagonists

Seven antagonists were investigated, each administered at a moderately high dose (Table 1). Subjects ($n = 64$) were first tested on 8 drug-free days and then alternately with saline and nicotine 0.1 mg/kg SC for 8 days. The 45 rats that met the selection criteria were each randomly assigned to one of two testing groups ($n = 23$ and 22, respectively). In group 1, the following pretreatment conditions were tested in combination with saline and nicotine challenge, within a 7×2 design (14 sessions/rat): saline (IP route), saline (SC route, tested twice), mecamylamine, SCH 39166, naloxone, propranolol. In group 2, the pretreatment conditions were sulpiride, rimonabant, and prazosin, each with its respective vehicle control (see Table 1 for details); each pretreatment was tested in combination with saline and nicotine challenge, within a 6×2 design (12 sessions/rat).

Experiment 2: Mecamylamine dose-response

The aim was to confirm that this nAChR antagonist abolished the nicotine REE, and to determine whether lower mecamylamine doses were also effective. Subjects ($n = 32$) were first tested on 8 drug-free days and then alternately with saline and nicotine 0.1 mg/kg SC for 8 days. A total of 17 rats were then tested in a 5×2 design (10 sessions/rat): pretreatment with saline (tested twice) and mecamylamine (0.1, 0.3 and 1 mg/kg SC), in combination with saline and nicotine challenge.

Experiment 3: SCH 39166 dose-response

Experiment 3.1 Here, lower doses of SCH 39166 were tested for selective inhibition of the nicotine REE. Subjects comprised the 32 rats that had completed Experiment 1 with the highest response rates. Before antagonist/nicotine testing, performance was verified by giving each rat two drug-free sessions, followed by one test each with either saline or nicotine (counterbalanced order); as a result, one rat was removed. The subsequent drug testing block ($n = 31$) followed a 4×2 design (i.e., 8 sessions/rat): pretreatment with SCH 39166 (0, 0.01, 0.03, and 0.1 mg/kg SC), in combination with saline and nicotine challenge.

Experiment 3.2 Here, SCH 39166 was tested in an even lower dose range. Subjects ($n = 32$) were first tested on 5 drug-free days and then alternately with saline and nicotine 0.1 mg/kg SC for 12 days. A total of 23 rats were then tested in a 5×2 design (10 sessions/rat): pretreatment with saline (tested twice), and SCH 39166 (0.003, 0.01, and 0.3 mg/kg SC), in combination with saline and nicotine challenge.

Experiment 4: Prazosin and naloxone at selected doses

This experiment sought to confirm whether prazosin and naloxone would reduce, or possibly abolish, the reinforcement enhancing effect of nicotine. Subjects ($n = 46$) were first tested on 8 drug-free days and then alternately with saline and nicotine 0.1 mg/kg SC for 12 days. A total of 32 rats were then selected for further testing in two consecutive blocks of drug testing, one featuring prazosin and the other naloxone. Each test block followed a 4×2 design (8 sessions/rat): pretreatment with saline (tested twice) and with two doses of antagonist, all in combination with saline and nicotine challenge. Testing followed a crossover design, such that each rat was tested with both prazosin and naloxone. To this end, rats were first randomly allocated to two groups. In the first block (Days 21–28), one group was tested with prazosin (0, 0, 1 and 2 mg/kg) while the other was tested with naloxone (0, 0, 1, 5 mg/kg). A one-week pause of testing followed, after which the selection criteria (described above) were reapplied in 10 once-daily alternating tests with saline and nicotine. Finally, in the second block of testing (Days 39–46), rats in each group were tested with the antagonist that they had not previously received.

Data analysis and statistics

Commercial software was used for statistical analyses (SYSTAT version 11, SPSS Inc., Chicago, IL, USA). The behavioral variables were the number of active and inactive lever presses per 60-min session. The number of reinforcers earned was identical to the number of active presses and hence is not reported. Where the same drug condition was tested more than once, data were averaged across sessions. In order to assess active vs. inactive lever preference in individual rats, paired *t* tests were used (with LEVER as the within-subjects factor and session serving as the experimental unit).

Table 2 Summary of statistical analysis.

Effect.	Dependent variable	Statistical test/comparison
Nicotine REE	Active presses	Paired *t* test, saline-nicotine vs. saline-saline
Antagonist/nicotine REE	Active presses	2-way ANOVA, ANTAG x NIC interaction
Antagonist alone (dose-response)	Active presses	1-way ANOVA
Antagonist alone (single dose)	Active presses	Paired *t* test, antagonist-saline vs. vehicle-saline

The main drug effects were analyzed as shown in Table 2. Since nicotine only consistently increases responding on the active lever in this behavioral task (Constantin and Clarke 2018), the nicotine REE was analyzed only in terms of active lever presses. Each analysis of variance (ANOVA) featured either one or two within-subject factors, i.e., NIC (nicotine vs. saline) and/or a factor representing pretreatment drug vs. vehicle comparison. For the repeated measures ANOVAs, Huynh-Feldt sphericity-corrected p values are reported. Inactive lever presses were only analyzed if active lever presses were found to be significantly altered by a drug. The active and inactive lever responses were not directly compared by ANOVA, as this would have violated the homoscedasticity assumption underlying this test. Outliers were identified by Grubb's test and were excluded from analysis. No correction was made for multiple comparisons, but the significance level (alpha, 2-tailed) was set at $p < 0.01$ for all analyses.

Results

In most subjects, responding on the active and inactive levers persisted across successive phases of each experiment, as documented for control and nicotine-alone test sessions in **Supplementary Table S2**. Across several weeks, the rate of active lever pressing during control sessions tended to decline. Thus, between the initial no-injection baseline phase and the block of drug testing, this measure changed by −21, − 26, − 36 and + 6% in Experiments 1–4, respectively. The main drug findings are summarized in Table 3.

Experiment 1: Single-dose antagonist tests

Out of 64 animals, 45 met the selection criteria and were randomly assigned to Group 1 or 2 (see Methods).

Group 1: Mecamylamine, SCH 39166, naloxone, and propranolol The effects of nicotine alone were assessed after both SC and IP saline pretreatment (Fig. 1**a**). After SC saline, nicotine significantly increased only active lever pressing (active $t_{22} = 5.54$, $p < 0.0001$; inactive $t_{22} = 1.74$, $p = 0.0955$). After IP saline, nicotine increased both active and inactive lever pressing (active $t_{22} = 3.66$, $p = 0.0014$; inactive $t_{22} = 3.08$, $p = 0.$ 0.0055). Mecamylamine (here, tested at 1 mg/kg) completely blocked the nicotine REE (MEC x NIC $F_{1, 22} = 21.66$, $p = 0.0001$) while having no effect on active lever pressing when given alone ($t_{22} = 0.35$, $p = 0.7314$; Fig. 1**a**). SCH 39166, tested only in a high dose (0.2 mg/kg), virtually abolished responding on both levers, even in the absence of nicotine (Fig. 1**a**). Naloxone (1 mg/kg) tended to blunt the nicotine REE, but not significantly (NAL x NIC $F_{1, 22} = 4.03$, $p = 0.0570$; Fig. 1**a**); naloxone also tended to reduce active lever responding when given alone ($t_{22=}2.67$, $p = 0.0140$). Propranolol (10 mg/kg) did not significantly affect the nicotine REE (PROP x NIC $F_{1, 22} = 0.0370$, $p = 0.8493$; Fig. 1**a**). When given alone, propranolol tended to increase active lever responses (active, $t_{22} = 2.50$, $p = 0.0202$; inactive $t_{22} = 1.95$, $p = 0.0640$).

Group 2: Sulpiride, rimonabant and prazosin Nicotine increased active lever presses after pretreatment with all three types of vehicle, i.e., acetic acid, DMSO/Tween and water (respectively $t_{21} = 5.53$, 8.09 and 7.63, $p < 0.0001$ for each; Fig. 1b–d). At the same time, nicotine did not significantly increase inactive lever presses ($t_{21} = 0.39$–1.99, $p = 0.02311$–0.6969). Sulpiride (40 mg/kg) did not significantly alter the nicotine REE (SULP x NIC $F_{1, 21} = 0.06$, $p = 0.8016$; Fig. 1**b**) while tending to inhibit active lever responding when given alone ($t_{21} = 2.34$, $p = 0.0293$). Rimonabant (3 mg/kg) decreased the nicotine REE (RIM x NIC $F_{1, 21} = 34.1$, $p < 0.0001$; Fig. 1**c**), but also exerted more general effects. In particular, rimonabant given alone decreased responding on both active and inactive levers (active $t_{21} = 3.30$, $p = 0.0034$; inactive $t_{21} = 3.76$, $p = 0.0011$). Prazosin (1 mg/kg) decreased the nicotine REE (PRAZ x NIC $F_{1, 21} = 8.26$, $p = 0.0091$; Fig. 1**d**), without affecting active lever responses when it was given alone ($t_{21} = 0.78$, $p = 0.4463$).

Experiment 2: Mecamylamine dose-response

Out of 32 animals, 17 met the selection criteria for drug testing. Nicotine given alone significantly increased active lever pressing (active $t_{16} = 4.26$, $p = 0.0006$, inactive $t_{16} = 0.20$, NS; Fig. 2). Mecamylamine dose-dependently inhibited the nicotine REE (linear trend on MEC x NIC $F_{1, 16} = 8.64$, $p = 0.0096$), completely suppressing it only at the highest dose (Fig. 2). Mecamylamine, when given alone, did not significantly alter active lever pressing ($F_{3, 48} = 3.07$, $p = 0.0637$).

Table 3 Summary of main findings.

Experiment.	Drug	Dose mg/kg tested	Effect on nicotine REE	Effect alone on lever pressing
1	Mecamylamine	1	Abolished	↔
2	Mecamylamine	0.1–1	Abolished (1 mg/kg)	↔
1	SCH 39166	0.2	Undeterminable	↓↓ (active and inactive)
3.1	SCH 39166	0.01–0.1	Abolished (0.01, 0.1 mg/kg)	↓ (0.03, 0.1 mg/kg, active and inactive)
3.2	SCH 39166	0.003–0.03	Abolished (0.01, 0.03 mg/kg)	↓ (0.03 mg/kg, active and inactive)
1	Naloxone	1	↓?	↓? (active)
4	Naloxone	1 and 5	Abolished (1, 5 mg/kg)	↔
1	Prazosin	1	↓	↔
4	Prazosin	1 and 2	↓? (1, 2 mg/kg)	↑ (1 mg/kg, active)
1	Sulpiride	40	↔	↓? (active)
1	Rimonabant	3	↓	↓ (active and inactive)
1	Propranolol	10	↔	↑? (active)

Notes: The significance level (i.e., alpha) was set at 1% (2-tailed). Arrows refer to effects of drugs on the nicotine REE or effects when given alone on active or inactive lever pressing: ↑ significant increase, ↓ significant decrease, ↔ no detectable effect. The symbols ↑? and ↓? refer to non-significant trends with $p = 0.05–0.01$, except naloxone in Experiment 1 ($p = 0.0570$) and prazosin in Experiment 4 (see main text). In dose-response studies, effective doses are shown in parentheses

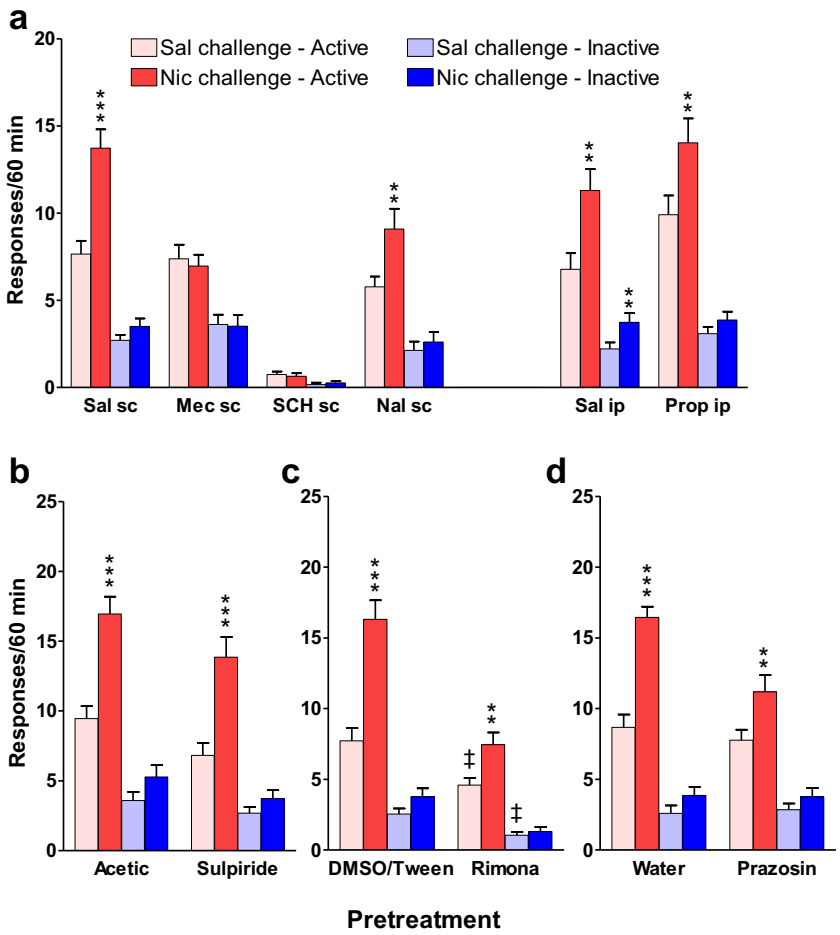

Fig. 1 Reinforcement enhancing effect of nicotine: single-dose tests with seven pretreatment drugs (Experiment 1). Panels a and b relate to Groups 1 and 2, respectively. The y-axis shows mean ± SEM active and inactive lever responses occurring during the 60-min session. Within a given group, each rat was tested under all combinations of pretreatment (i.e., drug and corresponding vehicle) and drug challenge (i.e., nicotine 0.1 mg/kg SC or saline). Abbreviations: Sal, saline; Mec, mecamylamine 1 mg/kg SC; SCH, SCH 39166 0.2 mg/kg SC; Nal, naloxone 1 mg/kg SC; Prop, propranolol 10 mg/kg IP; Acetic, dilute acetic acid vehicle; Sulp, sulpiride 40 mg/kg SC; DMSO/Tween, DMSO/Tween-80 vehicle; Rimona, rimonabant 3 mg/kg IP; Prazosin, prazosin 1 mg/kg IP. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. corresponding saline challenge, ‡$p < 0.01$ vs. corresponding vehicle pretreatment (paired t tests, $n = 22–23$ rats)

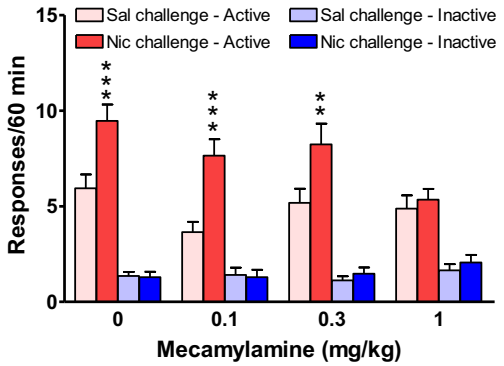

Fig. 2 Inhibition of nicotine-induced reinforcement enhancement by mecamylamine (Experiment 2). Each rat was tested under all eight conditions, i.e., mecamylamine (0, 0.1, 0.3 or 1 mg/kg SC) given 20 min before nicotine (0.1 mg/kg SC) or saline. The y-axis shows mean ± SEM active and inactive lever responses occurring during the 60-min session. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to corresponding saline challenge (paired *t* tests, $n = 17$ rats)

Experiment 3: SCH 39166 dose-response

Experiment 3.1 Higher dose range of SCH 39166 As described in Methods, subjects were the 31 highest-responding rats from Experiment 1. Nicotine, administered alone, significantly increased active lever pressing (active $t_{30} = 7.40$, $p < 0.0001$, inactive $t_{30} = 2.03$, NS; Fig. 3**a**). The nicotine REE appeared blocked at the lowest antagonist dose tested (Fig. 3**a**), yet a residual nicotine effect was detected at the middle dose (0.03 mg/kg: $t_{30} = 3.73$, $p = 0.0008$). When given alone, SCH 39166 dose-dependently reduced pressing on both active and inactive levers (active lever, linear trend $F_{1, 30} = 110.7$, $p < 0.0001$, inactive lever, linear trend $F_{1, 30} = 42.2$, $p < 0.0001$). In the absence of nicotine, the highest dose of the antagonist profoundly reduced responding on both levers, and even the lowest antagonist dose (0.01 mg/kg) tended to reduce active lever pressing ($t_{30} = 2.24$, $p = 0.0327$).

Experiment 3.2 Lower dose range of SCH 39166 Out of 32 animals, 23 met the selection criteria for drug testing, but subsequently one rat was identified as an outlier and hence excluded from the analysis. Nicotine alone significantly increased active lever presses (active $t_{21} = 6.12$, $p < 0.0001$, inactive $t_{21} = 0.24$, NS; Fig. 3b). The active lever data showed a clear overall interaction between SCH 39166 and nicotine ($F_{3, 63} = 5.20$, $p = 0.0061$), and a follow-up analysis of individual antagonist doses (compared with saline pretreatment) revealed significant SCH 39166 x nicotine interactions at 0.01 and 0.03 mg/kg (respectively: $F_{1, 21} = 24.30$ and 15.57, $p =$ 0.0001 and 0.0007). Nicotine did not significantly increase active lever presses at any dose of SCH 39166 ($t_{21} = 0.59$–1.62, NS; Fig. 3b).

When given alone, SCH 39166 dose-dependently reduced pressing on both active and inactive levers (active lever, linear trend $F_{1, 21} = 20.58$, $p = 0.0002$; inactive lever, linear trend $F_{1, 21} = 28.9$, $p < 0.0001$; Fig. 3b). However, in the absence of nicotine, only the highest antagonist dose (0.03 mg/kg) significantly reduced active lever presses ($t_{21} = 4.16$, $p = 0.0004$); this dose also inhibited inactive lever responding ($t_{21} = 4.98$, $p = 0.0001$). Even at this high dose, the animals continued to respond preferentially on the active lever (Fig. 3b).

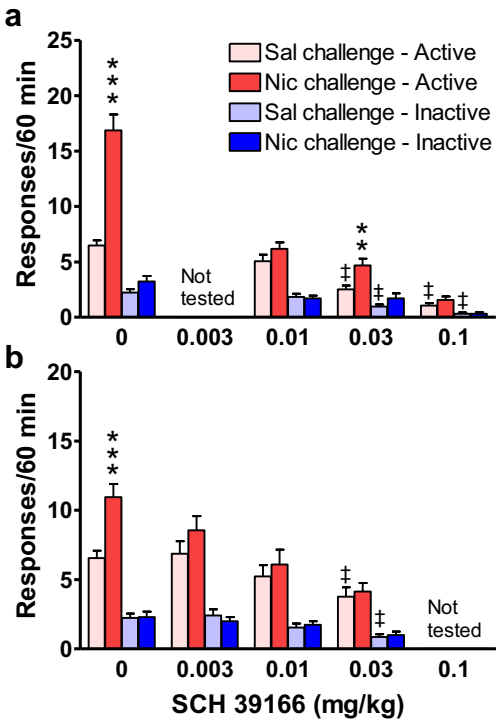

Fig. 3 Inhibition of nicotine-induced reinforcement enhancement by the DA D1-like receptor antagonist SCH 39166 (Experiment 3). Panels a and b represent Experiments 3.1 and 3.2, respectively. Within each experiment, each rat was tested under all eight conditions, i.e., SCH 39166 (at doses shown, SC route), given 20 min before saline or nicotine (0.1 mg/kg SC). The y-axis shows mean ± SEM active and inactive lever responses occurring during the 60-min session. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. corresponding saline challenge, ‡p < 0.01 vs. corresponding saline pretreatment (paired t-tests, $n = 31$ and 22, panels a and b, respectively)

Experiment 4: Prazosin and naloxone at selected doses

This experiment comprised two blocks of drug testing, which were separated by a 10-day period of nicotine and saline tests (see Methods). Out of 46 rats that started the experiment, a significant number failed to meet the selection criteria, and consequently prazosin and naloxone were tested in 25 and 20 rats, respectively.

Prazosin. Given alone, nicotine selectively increased active lever pressing (active $t_{24} = 9.55$, $p < 0.0001$; inactive $t_{24} =$ 2.38, NS). Prazosin given alone increased active lever presses at the lower dose (1 mg/kg: $t_{24} = 3.45$, $p = 0.0021$). Prazosin appeared to inhibit the nicotine REE, as seen in Experiment 1, but less clearly. Thus, although nicotine did not significantly increase active lever pressing at either dose of prazosin ($t_{24} =$

0.93 and 1.58, NS; Fig. 4a), the prazosin x nicotine interaction was not significant ($F_{2, 48} = 2.14$, $p = 0.1286$).

Naloxone. When given alone, nicotine increased active lever ($t_{19} = 6.14$, $p < 0.0001$), but not inactive lever pressing ($t_{19} = 0.71$ $p = 0.4890$; Fig. 4b). The nicotine REE was disrupted by naloxone (naloxone x nicotine interaction $F_{2, 38} = 8.33$, $p = 0.0010$). Nicotine did not significantly increase active lever responding at either dose of naloxone (respectively: $t_{19} = 1.56$ and 0.53, NS), and the nicotine REE appeared completely blocked at the higher dose of naloxone (Fig. 4b). Naloxone given alone did not significantly alter active lever pressing ($t_{19} = 1.45$ and 1.01, NS).

Discussion

Novel findings

A key feature of the present study is the use of a relatively low dose of nicotine (0.1 mg/kg SC), chosen to produce peak within-session plasma levels comparable to between-cigarette levels in habitual smokers (Constantin and Clarke 2018). Such a low dose has rarely been used in pharmacological studies of the nicotine REE (Tobey et al. 2012; Wing and Shoaib 2010). As expected, acute nicotine administration enhanced the effectiveness of the primary sensory reinforcer in a behaviorally specific manner (Constantin and Clarke 2018; Wright et al. 2018). We demonstrate here for the first time that the nicotine-induced enhancement of a primary visual reinforcer can be inhibited or abolished by antagonists of D1-like dopaminergic, opioid, CB1 cannabinoid, and alpha1-adrenergic receptors, independent of motor disruption. As noted below, some of these observations appear to be novel for any type of reinforcer or nicotine dose.

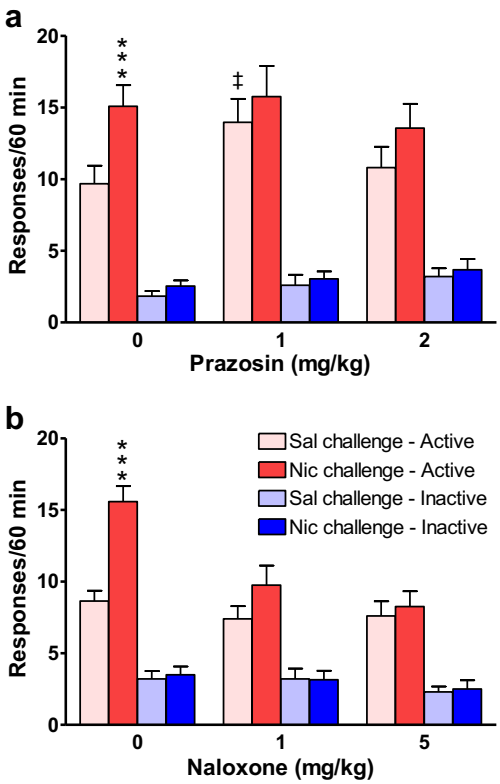

Fig. 4 Reinforcement enhancement by nicotine: effects of prazosin and naloxone (alpha1-adrenergic and opioid antagonist, respectively; Experiment 4). Each rat was tested under all pretreatment conditions. Prazosin or vehicle was given IP (20 min pre-session), whereas naloxone or vehicle was given SC (10 min pre-session), and these pretreatments were followed by acute nicotine (0.1 mg/kg SC) or saline challenge. The y-axis shows mean ± SEM active and inactive lever responses occurring during the 60-min session. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. corresponding saline challenge, ‡prazosin alone vs. vehicle (paired *t* tests, $n = 25$ and 20, panels a and b, respectively)

Methodological aspects

Our operant task differed in several respects from procedures used by other groups. Relevant details are discussed further elsewhere (Constantin and Clarke 2018) and are briefly summarized as follows. First, the continuous reinforcement (FR1) schedule included lever retraction after each response, preventing any response extinction during timeout periods. Second, our rats were neither food-restricted nor trained in advance to lever-press for food; while this may have resulted in lower response rates, it simplified data interpretation. Third, our primary reinforcer (a 3-s cue light) was probably weaker than the compound visual stimuli used in many other studies. Nevertheless, as in our previous studies, most subjects continued to lever-press for the visual stimulus at a similar rate over several weeks. Fourth, this visual stimulus is unlikely to have acquired significant secondary reinforcer properties through association with nicotine, for multiple reasons (Constantin and Clarke 2018). Lastly, even after several weeks of daily testing, responding does not become habitual (Wright et al. 2018).

In our two previous studies, almost all rats successfully completed testing (Constantin and Clarke 2018; Wright et al. 2018). In the present study, in contrast, the attrition rate was significant. For example, in Experiment 1, only 45 of the 64 rats met the three selection criteria (preferential active lever responding with a minimum of 5 presses/session, increased by nicotine). Since the daily testing procedure was the same in all three studies, the elevated attrition rate is possibly related to specific batches of rats, individual experimenters, or other unidentified factors.

In assessing possible drug effects on the nicotine REE, competing explanations were considered, as follows. First, possible drug-induced motor disruption was identified by decreases in active and inactive lever responses, in the absence of nicotine, such was the case only for rimonabant and for the higher doses of SCH 39166. Second, we also verified that no

pretreatment drug when given alone appreciably blunted the reinforcing impact of the visual stimulus or impaired attention.

Individual drugs and receptor targets

Mecamylamine (nicotinic ACh receptor antagonist)

Mecamylamine abolished the nicotine REE at the highest dose tested (1 mg/kg), without altering response rates when given alone. The same overall result has previously been reported with both primary or conditioned reinforcers, studied with higher SC doses (i.e., 0.3–0.6 mg/kg) of nicotine (e.g., Guy and Fletcher 2013; Ivanová and Greenshaw 1997; Kirshenbaum et al. 2014; Olausson et al. 2004; Palmatier et al. 2009). Mecamylamine, given at 1 mg/kg, would likely antagonize a variety of CNS and ganglionic nAChR subtypes (Papke et al. 2001) but without inhibiting NMDA-type glutamate receptors (Clarke et al. 1994). Since mecamylamine did not itself enhance reinforced responding, it appears that nicotine exerted its REE via nAChR stimulation rather than desensitization. Here, α4β2* and/or α6* nAChR subtype(s) are candidates (Barrett et al. 2018; Guy et al. 2014; Levin et al. 2012; Spiller et al. 2009; Tobey et al. 2012).

SCH 39166 (D1-like receptor antagonist)

The commonly used D1-like receptor antagonist SCH 23390 has been shown to reduce or block reinforcement enhancing effects of high-dose nicotine, in studies of brain stimulation reward (Harrison et al. 2002), sign-tracking (Palmatier et al. 2014), and conditioned reinforcement (Guy and Fletcher 2014). SCH 23390 and nicotine have also been tested in combination with a primary visual reinforcer (Barrett et al. 2016), but the results do not clearly indicate whether SCH 23390 blunted the nicotine REE or disrupted responding more generally. In the present study, as well as using a lower dose of nicotine, we tested a different D1-like antagonist, SCH 39166, which unlike SCH 23390 lacks significant 5-HT receptor affinity (Wamsley et al. 1991). SCH 39166 blocked the nicotine REE at two intermediate doses (0.01 and 0.03 mg/kg), independent of response suppression.

Sulpiride (D2-like receptor antagonist)

For unclear reasons, D2-like receptor antagonists have produced mixed results in nicotine REE studies in rats, as follows. In ICSS studies, the reward threshold-lowering effect of nicotine was reduced or blocked by a D2-selective dose of haloperidol (Ivanová and Greenshaw 1997) and by the D3 DA antagonist SB-277011A (Pak et al. 2006), whereas the D2/D3 antagonist eticlopride was ineffective even at high doses (Harrison et al. 2002). Where sensory reinforcers have been used, eticlopride blocked nicotine-induced enhancement of a conditioned audiovisual reinforcer (Guy and Fletcher 2014), but comparable doses did not attenuate a REE using a primary visual reinforcer (Barrett et al. 2016). Tested at higher doses, eticlopride also attenuated enhanced sign-tracking by the same dose of nicotine (Palmatier et al. 2014).

The present study differed from previous D2-like antagonist studies, not only in using a lower dose of nicotine (i.e., 0.1 mg/kg vs. 0.25–0.6 mg/kg SC) but also in using the D2-like antagonist sulpiride. The S-isomer of sulpiride that we used is highly selective for D2-like DA receptors (i.e., D2, D3 and D4 receptors) over D1-like DA receptors (Seeman and Van Tol 1994). Sulpiride was chosen from among D2-like antagonists because even high doses produce little if any motor disruption (Morgenstern et al. 1983). In the present study, sulpiride did not detectably inhibit the nicotine REE. Importantly, our test dose (40 mg/kg) is reportedly effective in other behavioral assays (Ogren and Fuxe 1988; Ogren et al. 1986), and in particular markedly affected nicotine IVSA even at a lower dose of 5 mg/kg (Sorge and Clarke 2009).

Naloxone (opioid receptor antagonist)

In the present study, naloxone (1 and 5 mg/kg) dose-dependently abolished the nicotine REE without appreciably affecting responding when given alone (Experiments 1 and 4). Previous relevant REE studies have yielded widely variable results. Thus, in a progressive ratio schedule motivated by sucrose reward, the nicotine REE was blocked by naloxone (3 mg/kg SC, Kirshenbaum et al. 2016), whereas the same antagonist, even in doses up to 16 mg/kg, failed to inhibit nicotine's ability to reduce brain stimulation reward thresholds (Huston-Lyons and Kornetsky 1992). Naltrexone, another opioid antagonist, also showed no interaction with nicotine in a task motivated by an audiovisual conditioned reinforcer (Guy et al. 2014), although naltrexone did block nicotine's ability to increase single-lever responding for a food reward (Corrigall et al. 1988). Variable results have been reported across studies of nicotine IVSA behavior (Ismayilova and Shoaib 2010).

Taken together, the above reports do not provide a coherent picture. Moreover, these studies all employed doses of nicotine (0.3–0.75 mg/kg SC, 0.03 mg/kg/infusion IV) that would have provided plasma nicotine levels beyond the normal smoking range (Constantin and Clarke 2018). In contrast, the present findings reveal an opioid receptor-dependent nicotine REE occurring at nicotine exposure levels likely to be smoking-relevant.

Prazosin and propranolol (alpha1 and beta-adrenergic receptor antagonists)

To our knowledge, a possible role of adrenergic receptors in the nicotine REE has been directly investigated in only one

published study (Palmatier et al. 2009). As in the present study, rats responded to obtain a primary visual reinforcer, and the nicotine REE was tested in combination with the beta1/beta2 antagonist propranolol (Baker 2005) and the highly selective alpha1 receptor antagonist prazosin (Balle et al. 2003). In this earlier report, neither propranolol nor prazosin detectably blunted the nicotine REE.

We tested a higher dose of propranolol (i.e., 10 mg/kg vs. 1 mg/kg), since propranolol can have graded effects in this dose range (Harris et al. 1996; Wright et al. 2012). Again, no REE-attenuating effect was detected. However, in the present study, prazosin (1 or 2 mg/kg) inhibited the nicotine REE (more clearly in Experiment 1, with a similar trend in Experiment 4). This result stands in contrast to the earlier negative report by Palmatier et al. (2009). Since both studies employed comparable doses of prazosin in combination with a primary visual reinforcer, it seems likely that the REE-attenuating effect of prazosin is more readily observed at a low nicotine dose (i.e., 0.1 mg/kg vs. 0.4 mg/kg used by Palmatier et al.). Although in our experiments prazosin did not convincingly abolish the nicotine REE, we avoided testing higher prazosin doses in view of reported sedative effects (Trovero et al. 1992). Finally, the present results offer a potential explanation for prazosin's reported ability to inhibit nicotine IVSA (Forget et al. 2010), given that this behavior is at least partly driven by a nicotine REE (Rupprecht et al. 2015).

Rimonabant (cannabinoid CB1 receptor inverse agonist)

Rimonabant is reported to inhibit nicotine IVSA (Cohen et al. 2002; Forget et al. 2009), but to our knowledge the present study provides the first direct evidence that rimonabant can inhibit a nicotine REE. In related work, the CB1 receptor antagonist AM251 was reported to block the facilitatory effect of low-dose nicotine on responding for a conditioned visual reinforcer and for food reward (Wing and Shoaib 2010). In the present study, rimonabant when given alone inhibited both active and inactive lever responding. A nonspecific inhibitory effect is also apparent in IVSA studies (Cohen et al. 2002; Forget et al. 2009), and its basis is unclear, particularly since our test dose of rimonabant (3 mg/kg IP) was reported not to inhibit locomotor activity (De Vry et al. 2004) or high-rate responding for brain stimulation reward (Deroche-Gamonet et al. 2001).

Study strengths and limitations

Study strengths include the following. First, the operant task offers several advantages (see Methodological aspects, above). Second, all experimental designs featured repeated measures and large sample sizes, and there was also some replication between experiments. Third, our nicotine dose provides plasma nicotine levels well within the typical smoking range (Constantin and Clarke 2018). Fourth, the use of a systemic route of antagonist administration should inform the selection of potential drug candidates, and facilitate comparison with any future studies in human subjects. Several limitations should also be acknowledged. First, only male rats were tested. Second, as discussed above, the attrition rate was higher than in our previous studies, for unknown reasons. Third, since each rat was tested under several drug conditions, drug history may have influenced drug responses on subsequent sessions; this possibility was mitigated through the use of Williams square designs, which provide counterbalancing for first-order carryover effects. Fourth, each receptor target was probed with only a single pretreatment drug. Lastly, our use of systemic drug administration served to identify neurotransmitters that contribute to the reinforcement-enhancing effect of nicotine but was not designed to reveal the psychological or neural processes underlying nicotine's reinforcement-enhancing effects. Nonetheless, these processes merit further investigation, for example, by using a reinforcer demand analysis (Barrett et al. 2016), as well as in future studies that use intracerebral drug administration and also either chemogenetic or optogenetic manipulation.

Conclusions and future directions

Here, we have identified several drugs which either inhibited or abolished a reinforcement-enhancing effect associated with smoking-relevant nicotine plasma levels: mecamylamine, SCH 39166, prazosin, naloxone and rimonabant. Questions for future animal work include the following. First, does nicotine exert its reinforcement-enhancing effects through a fixed set of nAChR subtypes, regardless of nicotine exposure level and type of reinforcer? Second, among the non-nicotinic receptors highlighted in the present work, are any situated downstream of the initial site of nAChR activation, playing a REE-*mediating* role? Alternatively, do any of these receptors play an REE-*enabling* role, requiring only normal levels of activation?

To date, none of our test drugs appear to have been directly investigated for REE-related effects in human subjects. Several issues arise from the present findings. First, does mecamylamine's ability to suppress cigarette smoking in clinical trials (Rose 2008) depend on an ability to inhibit reinforcement-enhancing effects of nicotine? A second issue relates to the opioid antagonists naloxone and naltrexone, which are reported to inhibit smoking behavior in only a subset of studies (see Epstein and King 2004). Given the present findings, could the negative reports in human subjects have been obtained in laboratory settings that offered less scope for reinforcement enhancement? A third issue concerns CB1 ligands: rimonabant itself possesses a serious adverse effect profile, but CB1 neutral antagonists appear safer (Nguyen

et al. 2019) and hence may be worth exploring further. Lastly, to our knowledge, it is not known how smoking behavior might be altered by selective D1-like dopaminergic and alpha1-adrenergic blockers. Hence, in summary, the present findings may encourage further studies in human smokers—particularly with SCH 39166 (i.e., ecopipam), prazosin, opioid antagonists, all of which appear well-tolerated upon acute administration.

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Compliance with ethical standards All research procedures were reviewed and approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Conflict of interest The authors declare that they have no conflict of interest.

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